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Biodiesel production from genetically engineered microalgae: Future of bioenergy in Iran

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A B S T R A C T

Current biomass sources for energy production in Iran include sewerage as well as agricultural, animal, food industry and municipal solid wastes, and are anticipated to account for about 14% of national energy consumption in near future. However, due to the considerable progress made in genetic engineering of various plants in Iran during the last decade and the great potentials of microalgae for biofuel production, these photosynthetic organisms could be nominated as the future source of bioenergy in Iran. An overview of status of bioenergy in the world and Iran as well as the potential and utilization of biomass in Iran is presented. The possibilities of increasing biofuel production through microalgal genetic engineering and the progress made so far are discussed. Biodiesel in the Iran and its future prospective is also reviewed, emphasizing the promising role of microalgae.

Contents

1. Introduction

Energy is essential for life and industry development, and the global economy actually runs on energy. Fossil fuels include about 88% of the global energy consumption, in which, oil, coal and neutral gas are the major fuels with 35%, 29% and 24% shares, respectively. The shares of nuclear energy and hydroelectricity are about 5% and 6% of the global primary energy consumption, respectively [1,2]. The application of fossil fuels as energy sources is unsustainable due to depleting limited resources and also due to the accumulation of greenhouse gases in the environment [3]. Greenhouse gases contribute to global warming and also have other impacts on the environment and human life. Recently, the potential threat of global climate change has increased, and fossil fuel usage has had the highest contribution to greenhouse gas emissions [4]. For instance, in

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2006, the fossil fuels-associated CO_2 emissions were about 29 billion tones [5]. In addition, because of the adsorption of one-third of the CO_2 emitted each year into the oceans, the water pH is turning gradually to more acidic which will affect marine ecosystem biodiversity [6,7].

Regarding the mentioned problems, therefore, there is a need for enhancement of global strategies for energy security and mitigation of CO_2-energy related emissions, including increased energy efficiency, increased use of clean fossil energy, and increased use of renewable energy [8]. Recently, the use of liquid biofuels (bioethanol and biodiesel), especially in the transport systems has shown rapid global growth. First generation biofuels have been mainly derived from food and oil crops including rapeseed oil, sugarcane, sugar beet, and maize as well as vegetable oils and animal fats using conventional technologies. The use of first generation biofuels has generated a lot of controversy, mainly due to their impact on the global food markets and on food security, especially with regards to the most vulnerable regions of the world economy [9,10]. It is important to note that at this time about 1% (14 million hectares) of the world's available arable land is being used for the production of biofuels which provides less than 1% of the global transport demands. It is obvious that increasing that share to anywhere near 100% is impractical owing to the severe impact on the world's food supply and the large areas of production land required [7,11]. This problem is more highlighted for Iran because of its environmental conditions, limitations on crop production, and the increasing need for food and therefore, growing energy crops is disputed [12]. The second generation biofuels comprise production fuels from the whole plant tissue, including energy crops or agricultural residues, forest harvesting residues or wood processing waste. The problem with this generation is the lack of efficient technologies for commercial exploitation of wastes as source for biofuel production.

Exploitation of microalgae as a source of bioenergy production could meet conditions required for a technically and economically viable biofuel resource, because it is cost competitive with petroleum fuels, requires no additional land use, enables air quality improvement (e.g. CO_2 sequestration), and requires minimal water use [13]. Many eukaryotic microalgae have the ability to store significant amounts of energy-rich compounds, such as triacylglycerol (TAG) and starch, that can be used for the production of several distinct biofuels, including biodiesel and ethanol [14]. However, several technical barriers, such as slow growth rate, low biomass production and low lipid contents, need to be overcome before microalgae can be used as an economically viable biofuel feedstock. In addition to conventional microalgae breeding, one of the most powerful tools is genetic engineering to engineer metabolic pathways in microalgae for more biomass and lipid production. Fortunately, significant advances in microalgal genomics and genetic engineering have been achieved during the last decade, and many methodologies were optimized for transformation of different kinds of microalgae [15,16].

This review underlines the advantages and problems of microalgal biodiesel production, role of genetic engineering as an ultimate solution to those problems and also the future prospective of microalgal biodiesel in Iran.

2. Global status of bioenergy

Because of concerns about the current first-generation of liquid biofuels, there is major interest in heading toward alternative systems of biofuel production, such as second-generation liquid biofuels based on lignocellulosic biomass. Although some pilot plants currently exist, economic production of second-generation biofuels still remains a dream to reach in the future. It has been predicted that their commercial production using biochemical processing will only begin in 10–20 years [17]. Such estimates vary considerably based on factors such as expected private sector investments, technology development and oil prices, but a minimum of five to ten years seems inevitable [18]. First-generation biofuels, on the other hand, are already being produced in significant commercial quantities in a number of countries. World production has increased constantly in recent years, with the United States and Brazil topping the producers chart, and only one type of fuel; bioethanol derived from sugar crops (e.g. Sugar cane, sweet sorghum and sugar beet) as well as cereals which contain starch (e.g. Maize and wheat). World ethanol production in 2009 reached 73.9 billion liters which shows more than 400% increase in comparison with that of 2000 (17 billion liters) [19]. Although it has been predicted that global ethanol production will continue to increase and in 2017, will double that of 2007 [20]. It also indicated that the United States and Brazil will remain as the largest ethanol producers through to 2017 followed by China, India and Thailand. On the other hand, however, FAO analysis indicates that, with the exception of bioethanol from sugar cane in Brazil, biofuels are generally not economically competitive with fossil fuels without subsidies [10].

Table 1
The agricultural residues in Iran.[a]

Type of crop wastes	Megatonne/year
Wheat wastes	7.5
Sugar cane wastes	4.3
Rice wastes	1.05
Barley wastes	0.6
Corn wastes	0.5
Potato wastes	1.5
Date wastes	0.9
Sugar beet wastes	0.25
Grape waste	0.9
Apple wastes	0.9

[a] Najafi et al., 2009.

Moreover, as for global biodiesel production, the report projects a slightly higher growth rates than for bioethanol to reach 24 billion liters by 2017 and that EU comprising over 50% of the global production will continue to be the largest biodiesel producer in 2017 and will be followed by Indonesia, Brazil, the United States and Malaysia, respectively. The currently available feedstock involved in biodiesel production include vegetable oils derived from oilseed crops, e.g. soybean, sunflower, jatropha, oil palm or rapeseed, used cooking oil and animal fat, e.g. pork lard and beef tallow [11].

3. Current status of bioenergy in Iran

Iran is ranked as one of the top countries that have many natural resources such as crude oil and natural gas. However, the utilization of these natural resources is limited and more importantly, the renewable liquid fuels are needed to eventually totally displace petroleum-derived transport fuels that contribute to global warming. On the other hand, the renewable energy resources in Iran are not efficiently utilized and biofuels are not effectively used to meet a variety of energy needs, including fuelling vehicles, generation electricity, etc. One-third of Iran's total land area can be utilized to produce crops if sufficient water is provided. However, in reality, only 12% of the total land area can be utilized for crop growing [12]. Therefore, growing energy crops despite the increasing need for food is disputed. To overcome the challenge, there seem to be two options available, first; managing the agricultural residues and energy production from these materials (bioethanol, biogas, etc.) which will provide a high share of energy supply in Iran (Table 1) [21]. The second option would be investing on non-food

crops such as jatropha and also microalgae for the production of biodiesel.

Jatropha is a perennial, deciduous, stem-succulent shrub, which produces seeds rich in oil easily convertible into biodiesel [22]. However, it does not seem to be the right alternative in Iran due to its optimum growing conditions. Maes et al. (2009) demonstrated that Jatropha is not common in regions with arid and semi-arid climates such as Iran and does not naturally occur in regions with rainfall of less than 944 mm year^{-1} [23]. In addition, Jatropha, despite the fact that it is largely undomesticated, needs resources like any crop to achieve high productivity. Therefore, Jatropha promotion combined with efforts to reduce financial risk would possibly lead to a competition for land with food crops or high carbon stocks and ultimately Jatropha would lose its acclaimed sustainability advantages [24]. On the hand, many microalgae genera and species are remarkably rich in oil, which can be converted to biodiesel using existing technology. Apart from biodiesel [5,25], microalgae can provide other different types of renewable biofuels such as methane produced by anaerobic digestion of the algal biomass [26] and photobiologically produced biohydrogen [27,28]. Presence of various saline lakes in Iran such as Lake Urmia, the third largest salt water lake on earth, which contain a wide and diverse range of microalgal species and more importantly the apparent possibility of establishing microalgae culture ponds in different areas of Iran especially southern regions, make the microalgal biodiesel production an achievable dream.

Considering the high importance of promoting renewable and alternative energy sources, the Iranian government in particular, the Vice Presidency of Science and Technology established the Iran Renewable Energy Initiative Council (IREIC) in 2008 [29]. The council was comprised of seven different departments i.e. hydropower, fuel cells and hydrogen, wind and waves energy, solar energy, biomass, geothermal energy and planning. Moreover, in the same year the council prepared and submitted the Iran's strategic document of renewable energies (ISDRE) which is passing its final ratification step by the Iranian government at present. In this document, biomass-derived energy has been highlighted and biomass has been divided into five categories, (1) agricultural waste; (2) animal waste; (3) municipal solid waste; (4) food industry waste and (5) sewerage. In addition, it has been anticipated that 14% of the national energy requirements equivalent to 137 million barrels of crude oil could be met by the energy derived from the above-mentioned sources [29].

Apart from the waste-oriented sources and as dictated by the ISDRE, the IREIC's Biomass department has also focused on the cultivation of non-edible sources of cellulose and oil for the production of bioethanol and biodiesel in the Southern regions of Iran, respectively. Having considered the serious efforts made by the Iranian government and the abundance of various sources of waste and non-edible plants, the future of bioenergy in Iran appears to be promising.

4. Biodiesel

In recent years, biodiesel has been receiving increasing attention not only as an alternative but also as a sustainable fuel [30,31]. It is used for diesel engines [32] and is becoming well-known as an environmentally friendly fuel due to its non-toxic and biodegradable characteristics [33]. The many advantages of biodiesel include high Cetane number (in the US at ≥47 while for diesel at ≥40), dissolved oxygen content (10–12% by weight), sulphur-free, better combustion process and improved emission profile of exhaust gas [34] and [35].

Any types of feedstock which contains free fatty acids and/or triglycerides such as vegetable oils, waste cooking oil, animal fats, and waste greases can be converted into biodiesel [36,37]. The most common way to produce biodiesel is by transesterification which takes place between a vegetable oil and an alcohol (methanol or ethanol) in the presence a catalyst (homogeneous or heterogeneous) or without the application of catalysts as in supercritical fluid method (SCM). Homogeneous catalysts i.e. basic (sodium hydroxide, potassium hydroxide and sodium methylate), and acidic catalysts (sulfuric acid) are necessary to boost the rate of the transesterification reaction. The type of homogenous used depends on the free fatty acids (FFA) content of the raw oil [38]. Heterogeneous catalysts such as metal oxides or carbonates [39], sulphonated amorphous carbon [40], heteropolyacid solid [41] and biocatalysts (specifically lipases) are commonly used as well.

5. Microalgal biodiesel

5.1. Microalgae

Algae are defined as any organisms which are plant-like and perform photosynthesis. Based on their morphology and size, algae are typically subdivided into two major categories; macroalgae and microalgae (microphytes). Macroalgae, for example kelps, are composed of multiple cells which organize to structures resembling roots, stems, and leaves of higher plants. Microalgae are commonly microscopic algae found in fresh water and marine systems. They are single-celled or colonial photosynthetic organisms which are attracting a growing amount of interest for industrial applications such as the production of special chemicals and nutritional supplements [42]. It has been estimated that microalgae produce approximately half of the atmospheric oxygen and use simultaneously the greenhouse gas carbon dioxide to grow photoautotrophically.

Microalgae are an enormously diverse group of primary producers, abundant in almost all ecosystems on earth ranging from marine and freshwater environments to desert sands, from hot springs to snow and ice [43]. It has been estimated that about 200,000-800,000 species exist of which about 35,000 species are described. Over 15,000 novel compounds originating from algal biomass have been chemically determined [44]. Their commercial importance in the food industry, aquaculture and as a natural source of high-value products include the production of carotenoids, long-chain polyunsaturated fatty acids, and phycocolloids. Due to their high physiological diversity microalgae have long been proposed as living "cell factories", as they produce an enormous variety of high value compounds for chemical industry, human diet and medicine, such as carotenoids, antioxidants, fatty acids, enzymes, polymers, peptides, toxins and sterols [44–47]. Many species are a source of natural products and have also been exploited for biotechnological applications [45,48]. Moreover, microalgae are of great ecological importance. These are important in CO_2 recycling through photosynthesis, which is comparable to that of higher plants in O_2-evolved systems [42]. As mentioned earlier, biodiesel from oil crops and bioethanol from sugarcane are being produced in increasing amounts as renewable biofuels worldwide, but their production in large quantities is not sustainable. An alternative is offered by microalgae which sustain many potential advantages.

5.2. Microalgal biodiesel: advantages, problems and perspectives

Microalgae were initially evaluated as a potential fuel source in the 1970s, but the production costs and technical limitations discouraged the commercial development of microalgae-based biodiesel production [49]. Subsequent studies which continued through the 1980s to present have shown that research and tech-

nology developments are enabling the commercial potential of microalgae to shift from microalgal food biotechnology to biofuel production [50].

Previously, it was shown that due to their highly efficient photosynthesis, microalgae have fast growth and as a result high biomass production [51,52]. For example, it is estimated that the biomass productivity of microalgae could be 50 times more than that of switch grass, which is the fastest growing terrestrial plant [50]. As microalgae are the primary producers of the food chain, they provide a wide range of different fatty acids which can be used for food, feed and biodiesel applications. Many microalgae species can produce substantial quantities of oil contents. The average oil content varies from 1 to 70% but under optimized conditions, some species can produce up to 90% oil of dry weight [7].

Biodiesel from microalgae sustains the following potential advantages:

- The potential production of oil by microalgae is higher than other oil crops due to use limited land resources without causing potential biomass deficit.
- They do not have adverse effect on traditional agriculture because they are not used as food and are not cultivated in arable lands.
- They can grow in extreme environments. They can also be cultivated using only seawater, CO_2, and sunlight.
- Finally, apart from biodiesel, they can be used for production a broad spectrum of biofuels and byproducts including biomethane and biohydrogen [26–28] and [42].

In spite of the mentioned many advantages, the major disadvantage of microalgae for biodiesel production is the low biomass concentration in the microalgal culture due to the light penetration limit, and also insufficient oil contents of microalgae cells. In addition to this, the small size of algal cells makes the harvest of algal biomasses relatively costly. Drying harvested algal biomass from high volume of water would be an energy-consuming process [50]. In total, microalgal farming facility compared to a conventional agricultural farm is more complicated and costly. Nevertheless, these problems are expected to be overcome or minimized by technology development.

Given the vast potential of microalgae as the most efficient primary producers of biomass, there is little doubt that they will eventually become one of the most important alternative energy sources. Some strategies have been suggested to develop biofuel production from microalgae as a cost effective industry. One of the new strategies is biorefinery or co-product strategy, in which a wide range of chemicals and biofuels from microalgae biomasses are produced by the integration of bioprocessing and environmental friendly chemical technologies [53]. Other strategies involve designing new photo-bioreactors with high photosynthesis efficiency [54–57], applying cost-effective technologies for biomass harvesting and drying, such as chemical flocculation [58], biological flocculation [59], filtration [60], centrifugation [61] and ultrasonic aggregation [62] for biomass harvesting, low-pressure shelf drying [63], drum drying [64], spray drying [65], fluidized bed drying [66], freeze drying [63] and refractance window dehydration technology [67] for biomass drying. However, the most efficient strategy suggested for enhancing biodiesel production from microalgae seems to be the genetic engineering of metabolic pathways associated with fatty acids production [68].

5.3. Microalgae genetic engineering

5.3.1. Technical progress in microalgae genetic engineering

The idea of microalgae genetic engineering to increase their valuable compounds is very alluring. Most of genetic improvement of microalgae has been focused on the production of useful materials applicable to the cosmetic and medical fields. Due to the absence of cell differentiation, it seems that genetic manipulations of microalgae should be a much simpler system compared with higher plants. In addition, allelic genes are usually absent because of the haploid nature of most vegetative stages of microalgae [69]. Nevertheless, the progress in the genetic engineering of microalgae was extremely slow and until last 10 years little work has been done by adopting a genetic engineering approach to improve the microalgae. The methodology development for microalgal transformation has advanced significantly in the last 15 years. Genetic modification and molecular tools have been developed for the green (Chlorophyta), red (Rhodophyta), and brown (Phaeophyta) algae; diatoms; euglenids; and dinoflagellates. More than 30 different strains of microalgae have been transformed successfully to date [14]. Most of the advances achieved in this area have been made on transformation of the green algae *Chlamydomonas reinhardtii*, as a model organism [70,71] for which stable genetic transformation at both the nuclear [72,73], and chloroplast [74] levels has been reported. Since 1998, successful transformation of some microalgae, including chlorophyte groups (*C. reinhardtii*, *Chlorella ellipsoidea*, *Chlorella saccharophila*; *C. vulgaris*, *Haematococcus pluvialis*, *V. carteri*, *Chlorella sorokiniana*, *Chlorella kessleri*, *Ulva lactuca*, *Dunaliella viridis*, and *D. salina*), heterokontophytes (*Nannochloropsis oculata*), Diatoms (such as *T. pseudonana*, *P. tricornutum*, *Navicula saprophila*, *Cylindrotheca fusiformis*, *Cyclotella cryptic*, and *Thalassiosira weissflogii*), phaeophytes (such as *Laminaria japonica* and *Undaria pinnatifada*), Rhodophytes (such as *C. merolae*, *Porphyra yezoensis*, *Porphyra miniata*, *Kappaphycus alvarezii*, *Gracilaria changii*, and *Porphyridium* sp.) Dinoflagellates (*Amphidinium* sp. and *Symbiodinium microadriaticum*), and euglenids (*Euglena gracilis*) have been reported [14].

A variety of transformation methods have been used to transfer DNA into microalgal cells. These methods include particle bombardment, agitation of a cell suspension in the presence of DNA and glass beads, agitation in the presence of DNA and silicon-carbide whiskers, electroporation, *grobacterium* infection, artificial transposons, viruses, and most recently *Agrobacterium*-mediated transformation. Among those, microprojectile bombardment has been developed for transformation of microalgae chloroplast [72,75–80]. The highest transformation rate has been achieved by the methods electroporation and also particle bombardment. The electroporation method is generally used in eukaryotic microalgae [78].

The major problems in developing microalgae genetic engineering are the low growth rate of microalgae and also the quantity of gene expression in the microalgae species [81]. Previous works showed that the expression efficiency of exogenous genes is mostly 0.1–0.9% of soluble proteins in host cells of microalgae [82], therefore, the most new works are focused on enhancing gene expression in microalgae as heterologous hosts. It has been proven that transformation frequency is highly dependent on the case study species [83]. Promoters have critical roles in successful gene expression and they can also regulate the temporal and spatial expression of a transgene. The 35S promoter from cauliflower mosaic virus (CaMV 35S) has been extensively used in plant biotechnology in most dicotyledonous and some monocotyledonous plants [84], however, it has not been shown a useful promoter in most algal species. The use of inducible promoters provides a better control of expression level [85,86] and their activities can be regulated by biotic and abiotic signals. Also, synthetic promoters constructed in the laboratory by combining the Hsp70A gene from *Chlamydomonas* with other promoters could be used for transgenes expression in microalgae [87].

Selectable marker genes are employed for the selection of transformed cells in culture media, as they promote the growth of transformed cells in the culture medium. Selectable markers are

normally antibiotic resistance genes added to the culture media, which confer resistance to chemical components. Antibiotic resistance markers are mainly based on bacterial antibiotic resistance genes which have been developed for *Chlamydomonas* transformation. In recent years, bacterial antibiotic-resistance gene markers which have become accessible include *Sh.ble, aadA* and *aphVIII* genes conferring resistance to the bleomycin family, spectinomycin and paromomycin, respectively [88–90]. Beside these antibiotic resistance markers, some other markers based on auxotrophic mutants have also been developed. Among them, NIA1 (formerly NIT1) selectable marker led to rescue the *nit1* mutants growing on nitrate as the only nitrogen source [75], while *arg7* enabled the *arg7* mutants to show arginin-independent behavior [72]. It has been proven that the *ble* selectable marker gene is suitable for most algae [91].

Algae often have an unusual codon usage which requires even further adjustments before a successful transformation is possible. Two bacterial genes, *aadA* and kanamycin resistance *aphA6* have been used for chloroplast transformation [92,93]. Moreover, several different reporter genes, such as *gus*, green fluorescent protein (GFP) and luciferase have been developed for gene expression and protein localization studies in microalgae [94,95]. The most commonly used reporter gene in microalge transformation was initially the β-glucuronidase. The *gus* gene allowed monitoring of transformation by either a simple histochemical method or a fluorescence-based, highly sensitive, quantitative assay and using *gus* for transgenic research will continue because of the simple and relatively inexpensive assays for monitoring its activity [96]. Chalfie et al. developed a reporter gene encoding the green fluorescent protein (GFP) for transgenic studies [97]. The lack of requirement for an exogenous substrate makes the GFP a favorite reporter for microalgae. Codon optimization of native GFP and luciferase reporter genes led to a high expression and detection of these transgenes [98]. Despite the GFP gene marker superiority, the need for an expensive microscope with fluorescence capability discourages its widespread use.

Finally, it is important to emphasize that unlike higher plants, microalgae have relatively fast growth rates, can achieve high cell densities under conditions of high light and aeration and can be grown in volumes of megalitres. In systems where genetic transformation is routine, primary transformants can be achieved in less than two weeks. Due to this matter that microalgae have simple structure, recombinant protein purification is a simpler process and costs are therefore lower than higher plants. In the case of green microalgae, these organisms are known as generally regarded as safe (GRAS)) category. It is clear that GM plants recently encountered to different Biosafety problems, including environmental and human health risks. Fortunately, because of containment for microalgae production, Biosafety problems are less than that in plants. In addition, cost of production is an over-riding consideration in developing bioreactor systems and estimates of costs of production of recombinant antibodies in microalgal bioreactor systems is less than that in plant and animal cells [99,100].

In near future current microalgal genomics projects will accelerate development of microalgae transformation methodologies and their subsequent exploitation. The coming years should prove to be an exciting time with important insights into algal biology, the creation of improved strains for specialized commercial applications as well as their potential use as bioreactors for the production of commercially valuable recombinant proteins and bioenergy [100].

5.3.2. Metabolic engineering of microalgae

Genetic studies on microalgae have been redirected mainly toward analysis of photosynthesis and metabolic pathways. Furthermore, microalgae were genetically modified to express high quality recombinant proteins, such as hormones or antibodies and also bioremediation of soils contaminated with heavy metals [101]. Genetically modified (GM) algae are especially suitable for containment and controlled growth in bioreactors under both phototrophic and heterotrophic conditions [48,102].

Metabolic engineering in microalgae has been applied for the synthesis of recombinant proteins and vaccines [99,103], the production of biohydrogen [104], and bioremediation of contaminated soil [101]. Moreover, most microalgae are unable to grow on exogenous glucose in the absence of light. They have been genetically modified for expression of glucose transporters (glut1 or hup1) to live in the dark along with glucose [102]. In addition, the metabolic engineering of microalgae for production of hydrogen has also been developed. Under sulphur-deficient conditions, the rate of O_2 production drops below that of the respiratory O_2 consumption, cultures become anaerobic and H_2 gas is generated. In a different study, through transformation of an antisense Crcp-Sulp in microalgae, the production of sulphate permease reduced [104]. Siripornadulsil et al. accomplished bioremediation of soil contaminated with heavy metals through expression of a foreign metallothionein or the *P5CS* gene in microalgae [101]. The *P5CS* gene increased cadmium tolerance and caused a marked increase in binding of the metal [105]. Recently, the production of antibodies and vaccines has been developed in microalgae [106]. For example, the production of VP1 protein fused to the cholera toxin B subunit has been performed in microalgae by chloroplast genetic engineering. In addition, synthesis of the antioxidant, the ketocarotenoid astaxanthine, has been performed in microalgae by introduction of the beta-c-4-oxygenase gene (*crt*O). Ethylene production also was demonstrated in the transgenic microalgae [107].

5.3.3. Microalgae genetic engineering for enhanced biofuel production

Recent "Omics" revolutions, including structural and functional genomics, transcriptomics, proteomics, metabolomics, and finally systems biology resulted in the identification of metabolic pathways, their regulations and optimization for enhanced biofuel production [108]. For example, high throughput genome sequencing of microalgae species revealed several pathways involved in their metabolic processes, such as inorganic carbon fixation, fermentation, selenoprotein expression, and vitamin biosynthesis, each of which can be used to improve the accumulation of targeted bioenergy carriers [109] and [110].

The application of genetic engineering to improve biofuel production in eukaryotic microalgae is in its infancy, but significant advances in the development of genetic manipulation tools have recently been achieved with microalgal model systems and are being used to manipulate central carbon metabolism in these organisms [14]. Recently, due to availability of genome databases and the previous studies, fatty acid biosynthesis pathways were characterized. It is obvious that biosynthesis of fatty acid occurs in the plastid of plants and microalgae before translocation to the cytoplasm for further assembly into diacylglyceride and triacylglyceride molecules. Many enzymes involved in biosynthesis of fatty acids are encoded by single genes and are targeted to the mitochondria where fatty acid precursors are required to produce essential cofactors for mitochondrial enzyme activity [111,112]. Plants fatty acids and triacylglycerides are fairly consistent, but the microalgal lipids are variable and frequently composed of triacylglycerides and polyunsaturated Fatty acids that are prone to undesirable oxidation reactions affecting downstream biofuel applications. Lipid biosynthesis in microalgae starts with the acetyl-CoA carboxylase (ACC), which catalyses the irreversible carboxylation of acetyl-coenzyme A (acetyl-CoA) to produce malonyl-CoA through its two catalytic activities, biotin carboxylase (BC) and carboxyltransferase (CT) [113]. Studies showed that the high oil content species of soybean, exhibits about a 2-fold

increase in *ACC* activity than low oil content species [114,115]. Roessler reported that the overexpression of the *ACC* gene isolated from the microalgae *Cyclotella cryptic* enhanced the enzyme activity to 2–3-folds [116].

Characterization of microalgal lipid metabolism is of great interest for the production of biodiesel fuel surrogates. Both the quantity and the quality of biodiesel precursors in microalgae are closely linked to how lipid metabolism is controlled. Lipid biosynthesis and catabolism, as well as pathways that modify the length and saturation of fatty acids, have not been as thoroughly investigated for algae as they have for terrestrial plants. However, many of the genes involved in lipid metabolism in terrestrial plants have homologues in the sequenced microalgal genomes. Therefore, it is probable that at least some of the transgenic strategies that have been used to modify the lipid content in higher plants will also be effective with microalgae [14].

One of the major methodologies for increasing lipid and starch accumulation in green algae and diatoms is nutrient stress. Previously, it was shown that under nitrogen deplete conditions, some green microalgae accumulate high levels of lipids as triacylglycerides, and phosphorus and sulphur deprivation induce the conversion of membrane phospholipids to neutral lipids [117–119].

There are some strategies to engineer biosynthesis of fatty acids in microalgae toward more compatible lipid profiles, including secretion of lipid to from the cells to the media, over-expression of major enzymes involved in biosynthesis of fatty acids, increasing the availability of precursor molecules such as acetyl-CoA, down-regulating the catabolism of fatty acids by inhibiting β-oxidation or lipase hydrolysis, altering saturation profiles through the introduction or regulation of desaturases and finally, optimization of length of fatty acid chains with thioesterases [14] and [112].Sheehan et al. in their study concluded that the overexpression of the *ACC* gene alone might not be sufficient to enhance the whole lipid biosynthesis pathway [120]. Blocking off competing pathways may also enhance lipid biosynthesis. β-oxidation is the principal metabolic pathway responsible for the degradation of fatty acids in eukaryotes [121]. It is therefore possible to enhance Triacylglycerol (TAG) biosynthesis by blocking this pathway. To enhance lipid biosynthesis, overexpression of more than one key enzyme in the TAG pathway is suggested by a few researchers [122,123]. Overexpression of transcription factors which interact with the enzyme may also enhance the rate of transcription of genes [124]. Furthermore, genetic engineering of the transcription factor may affect up- or down-regulation of genes responsible for lipid synthesis as well [124]. Reik et al. (2007) studied the over-expression of a ZFP TF (Zinc-finger protein transcription factors) that binds a DNA sequence within the promoter [125]. They found out that it led to an enhanced lipid synthesis [53,125]. However, in spite of all these improved production of valuable products and bioactive compounds, there are still at least two major hindrances associated with microalgae genetic engineering; one is the growth rate of microalgae, and the other is the expression efficiency of exogenous genes in microalgae [81].

One of the most costly downstream processing steps in biodiesel production using microalgal feedstock is the extraction of fuel precursors from the biomass. One possible solution is to manipulate the biology of microalgal cells to allow for the secretion of lipids into the growth medium. There are in fact several pathways in nature that lead to secretion of hydrophobic compounds, including TAGs, free fatty acids, alkanes, and wax esters. Previous works on the secretion of free fatty acids in yeast have shown that inactivation of genes involved in β-oxidation and fatty acyl-CoA synthetase activity resulted in fatty acid secretion in some instances [126,127]. These genes were identified to have a function in fatty acid secretion in yeast. Similar screening methods could be utilized to identify microalgae that have the ability to secrete fatty acids [14].

Table 2
Comparison of some sources of biodiesel.

Crop	Oil yield (L/ha)[a]	Land area needed (M ha)[b]	Percent of existing Iran cropping area[b]
Corn	172	106	65
Soybean	446	41	25
Canola	1190	15.3	9
Jatropha	1892	9.6	6
Microalgae[c]	136,900[d]	0.13	0.08
Microalgae[e]	58,700[d]	0.31	0.19

[a] Chisti, 2007.
[b] For meeting 50% of all transport fuel needs of Iran.
[c] 70% oil (by weight) in biomass.
[d] Oil yields based on biomass productivity in photobioreactors.
[e] 30% oil (by weight) in biomass.

There are several identified pathways for the secretion of lipophilic compounds. These include the secretion of TAG-containing very-low-density lipid (VLDL) vesicles from hepatocytes, TAG-containing vesicles from mammary glands, and the ATP-binding cassette (ABC) transporter-mediated export of plant waxes, which consist of many types of hydrocarbons. In addition to cellular export pathways, there are also known pathways for intracellular transport of fatty acids between organelles, including import of fatty acids into mitochondria and peroxisomes for β-oxidation, and it may be possible to utilize such pathways for the export of lipids. Several key genes are known for these pathways, and transgenic expression of ABC transporters has been used to enable drug transport, resulting in resistance. However, the successful transgenic expression and utilization of lipid secretion pathways to secrete molecules suitable for biofuel production remain largely to be demonstrated. It was shown that deletion and over-expression of a wide range of genes, such as those encoding apolipoprotein E (ApoE), microsomal triglyceride transfer protein (MTP), triacylglycerol hydrolase (TGH), and arylacetamide deacetylase (AADA), significantly affect the secretion of very low density lipids [128,129].

One of the most straightforward approaches for enhancing the secretion of lipids from microalgae is the use of ABC transporters. ABC transporters mediate the secretion of different kinds of plant waxes. Previous works showed that transgenic expression of ABC transporters has resulted in transport of various compounds, including kanamycin, cholesterol, and sterols [130–132].

6. Biodiesel in Iran; future prospective

Replacing all the diesel fuel consumed in Iran with biodiesel will require 36.5 million m^3 of biodiesel annually at the current rate of consumption. Oil crops, waste cooking oil and animal fat cannot realistically satisfy this demand. For example, meeting only half the existing Iran diesel fuel needs by biodiesel would require unsustainably large cultivation areas for major oil crops. As demonstrated in Table 2, using the average oil yield per hectare from various crops, the cropping area needed to meet 50% of Iran diesel fuel needs is calculated in column 3 and in column 4, this area is expressed as a percentage of the current total cropping area of Iran (1,636,000 km^2) [133]. If Jatropha, a high-yielding oil crop can be grown, 6% of the total cropland will need to be devoted to its cultivation to meet only 50% of the diesel fuel needs but as previously mentioned, Iran's climate does not meet the optimum growing requirements of Jatropha. Therefore, oil crops are not anticipated to significantly contribute to replacing petroleum-derived liquid fuels in the near future in Iran. This scenario changes drastically, if microalgae are used to produce biodiesel. Between 0.08 and 0.19% of the total Iran's cropping area would be sufficient for producing algal biomass that satisfies 50% of the diesel fuel needs (Table 2).

Table 3
Oil content of some microalgal species.[a]

Microalgal species	Oil content (% dry wt)
Botryococcus braunii	25–75
Chlorella sp.	28–32
Crypthecodinium cohnii	20
Cylindrotheca sp.	16–37
Dunaliella primolecta	23
Isochrysis sp.	25–33
Monallanthus salina	>20
Nannochloris sp.	20–35
Nannochloropsis sp.	31–68
Neochloris oleoabundans	35–54
Nitzschia sp.	45–47
Phaeodactylum tricornutum	20–30
Schizochytrium sp.	50–77
Tetraselmis sueica	15–23

[a] Chisti, 2007.

In prospect of Table 1, microalgae come into view as the only source of biodiesel in Iran that has the potential to completely replace petroleum-derived diesel. Unlike other oil crops, microalgae grow extremely rapidly and many are remarkably rich in oil. Microalgae normally double their biomass within 24 h and this climbs down to as short as 3.5 h during exponential growth with the oil content varying between 15 to 75% by weight of dry biomass depending on the species (Table 3) [53,134,135]. Therefore, given the intrinsic advantages of microalgae, and the currently existing restraints of using other oil crops, microalgae genetic engineering would help to visualize more the economic production of biodiesel in Iran. Moreover, currently highly facilitated research centers, e.g. Agricultural Research Institute of Iran (ABRII) and Tarbiat Modares University (TMU) are conducting extensive research projects on various aspects of biofuels production in particular biodiesel.

On the other hand, due to the importance of genetic engineering approaches especially in the agriculture sector, during the last decade many highly facilitated and equipped labs have been established in Iran. Research experiences on plant genetic engineering in Iran dates back to more than 10 years ago when the first GM rice was produced in 1997. Since then, different kinds of GM crops, including rice, rapeseed, potato, cotton, alfalfa, tobacco, corn and sugar beet have been produced, and currently some are being evaluated by greenhouse and field trials [136–151]. In addition to the mentioned GM plants, some other GM plants such as wheat, barley, soybean, pear and lime are in the pipeline. These mark Iranian research institutes as capable candidates to accomplish projects on microalgae genetic engineering in order to finally meet the growing biodiesel requirements of the country in future.

Another important issue is the recent parliamentary and government approval (2009) and implementation of Iran's National Biosafety Law (INBL). Based on the Law, production and release of transgenic organisms are free in Iran provided that they pass all the biosafety requirements imposed by the INBL. This would facilitate the development and adaptation of transgenic microalgae in Iran. From the cultivation point of view, it is also important to note that apart from free and unlimited access to saline water and abundant sunshine and as mentioned earlier, there are many saline lakes (e.g. Lake Uremia, Lake Qom, Lake Gave Khooni, etc.) containing proven and wide range of different kinds of microalgae which could be used as sources of oil production [152].

7. Conclusions

Large-scale utilization and export of Iran's crude oil and natural gas in near future will be limited and the renewable liquid fuels will be heavily needed to eventually totally displace petroleum-derived transport fuels that also contribute to global warming. Because of the climate and geographical problems, only 12% of the total land area of Iran can be utilized for crop growing, therefore, growing energy crops despite the increasing need for food is not possible in Iran. To overcome the challenge, there seem to be two options available, first; managing the agricultural residues and energy production from these materials (bioethanol, biogas, etc.), the second option would be investing on non-food crops e.g. Jatropha and microalgae for the production of biodiesel. Because of the high needs of jatropha for water (more than 944 mm $year^{-1}$ rainfall), it does not seem to be the right alternative in Iran. On the other hand, however, many microalgae genera and species are remarkably rich in oil, which can be converted to biodiesel using existing technology. Apart from biodiesel, microalgae can serve as a source for other different types of renewable biofuels such as bioethanol, methane and biohydrogen. These potentials and the possibility of achieving economic production of microalgae biodiesel are more highlighted in Iran due to, (1) the presence of different saline lakes in Iran, containing various species of microalgae, (2) possibility of establishing microalgae culture ponds in different areas of Iran due to unlimited access to saline water and sunshine, (3) presence of highly efficient genetic engineering technologies in the world and good capacity building as well as the gained experiences in plant genetic engineering in Iran within the last decade, and (4) string government support.

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References

[1] British Petroleum, BP Statistical Review of World Energy; 2009.
[2] Brennan L, wende PO. Biofuels from microalgae—a review of technologies for production, processing, and extractions of biofuels and co-products. Renew Sust Energ Rev 2010;14:557–77.
[3] Khan SA, Rashm R, Hussain Z, Prasad S, Banerjee UC. Prospects of biodiesel production from microalgae in India. Renew Sust Energ Rev 2009;13:2361–72.
[4] Ugarte DG, Walsh ME, Shapouri H, Slinsky P. The economic impacts of bioenergy crop production in US agriculture, USDA Agricultural Economic Report, 816; 2003. p. 41.
[5] Banerjee A, Sharma R, Chisti Y, Banerjee UC. Botryococcus brauni: a renewable source of hydrocarbons and other chemicals. Crit Rev Biotechnol 2002;22:245–79.
[6] Ormerod WG, Freund P, Smith A, Davison J. Ocean storage of CO. IEAgreenhouse gas R&D programme. UK: IEA; 2002.
[7] Mata TM, Martins AA, Caetano NS. Microalgae for biodiesel production and other applications: a review. Renew Sust Energ Rev 2010;14:217–32.
[8] Sorest JP. Climatic change: solutions in sight, a Dutch perspective. Delft: Energy Policy Platform; 2000.
[9] FAO, Sustainable bioenergy: a framework for decision makers. United Nations Energy; 2007.
[10] FAO, The state of food and agriculture 2008: biofuels: prospects, risks and opportunities. Available from: http://www.fao.org/publications/sofa-2008/en [accessed 17.07.2010].
[11] IEA, Biofuels for transport, International Energy Agency. Available from http://www.iea.org/textbase/nppdf/free/2004/biofuels2004.pdf; 2004 [accessed 17.07.2010].
[12] Najafi G, Ghobadian B, Tavakoli T, Yusaf T. Potential of bioethanol production from agricultural wastes in Iran. Renew Sust Energ Rev 2009. doi:10.1016/j.rser.2008.08.010.
[13] Wang B, Li Y, Wu N, Lan C. CO2 bio-mitigation using microalga. Appl Microbiol Biotechnol 2008;79:707–18.
[14] Radakovits R, Jinkerson RE, Darzins A, Posewitz MC. Genetic engineering of algae for enhanced biofuel production. Eukaryot Cell 2010;9:486–501.
[15] Dismukes GC, Carrieri D, Bennette N, Ananyev GM, Posewitz MC. Aquatic phototrophs: efficient alternatives to land-based crops for biofuels. Curr Opin Biotechnol 2008;19:235–40.

[16] Pienkos PT, Darzins A. The promise and challenges of microalgal-derived biofuels. Biofuels Bioprod Bioref 2008;3:431–40.
[17] Larson ED. Biofuel production technologies: status, prospects and implications for trade and development. United Nations Conference on Trade and Development (UNCTAD). Available from: http://www.unctad.org/en/docs/ditcted200710_en.pdf; 2008 [accessed 17.07.2010].
[18] Rotman D. The price of biofuels. Technol Rev January/February, 2008.
[19] Renewable Fuels Association. 2010 Ethanol Industry Outlook: Climate of Opportunity. Available from: http://www.ethanolrfa.org/industry/outlook/RFAoutlook2010_fin.pdf; 2010 [accessed 17.07.2010].
[20] OECD-FAO agricultural outlook 2008–2017, OECD-FAO; 2008.
[21] Agricultural ministry of Iran, the agro-ecological zones, crop production statistics. Available from http://www.maj.ir/English/Main/Default.asp; 2010 [accessed 17.07.2010].
[22] Foidl N, Foidl G, Sanchez M, Mittelbach M, Hackel S. *Jatropha curcas* L. as a source for the production of biofuel in Nicaragua. Bioresour Technol 1996;58:77–82.
[23] Maes WH, Achten WMJ, Reubens B, Samson R, Muys B. Plant–water relationships and growth strategies of *Jatropha curcas* L. saplings under different levels of drought stress. J Arid Environ 2009;73:877–84.
[24] Achten WMJ, Maes WH, Aerts R, Verchot L, Trabucco A, Mathijs E, et al. Jatropha: From global hype to local opportunity. J Arid Environ 2010;74:164–5.
[25] Gavrilescu M, Chisti Y. Biotechnology—a sustainable alternative for chemical industry. Biotechnol Adv 2005;23:471–99.
[26] Spolaore P, Joannis-Cassan C, Duran E, Isambert A. Commercial applications of microalgae. J Biosci Bioeng 2006;101:87–96.
[27] Fedorov AS, Kosourov S, Ghirardi ML, Seibert M. Continuous H_2 photoproduction by *Chlamydomonas reinhardtii* using a novel two-stage, sulfate-limited chemostat system. Appl Biochem Biotechnol 2005;121–124:403–12.
[28] Kapdan IK, Kargi F. Bio-hydrogen production from waste materials. Enzyme Microb Technol 2006;38:569–82.
[29] Iran Renewable Energy Initiative Council Website. http://www.reic.ir/ [accessed 17.07.2010].
[30] Peng LB, Gaanty PM, Shafida AH. Biodiesel production via transesterification of palm olein using waste mud crab (Scylla serrata) shell as a heterogeneous catalyst. Bioresour Technol 2009;100:6362–8.
[31] Rajendra M, Jena PC, Raheman H. Prediction of optimized pretreatment process parameters for biodiesel production using ANN and GA. Fuel 2009;88:868–75.
[32] Haas MJ. Improving the economics of biodiesel productionthrough the use of low value lipids as feedstocks:vegetable oil soap stock. Fuel Process Technol 2005;86:1087–96.
[33] Ellis N, Guan F, Chen T, Poon C. Monitoring biodiesel production (transesterification) using in situ viscometer. J Chem Eng 2008;138:200–6.
[34] Keskin A, Guru M, Altıparmak D. Influence of tall oil biodiesel with Mg and Mo based fuel additives on diesel engine performance and emission. Bioresour Technol 2008;99:6434–8.
[35] Lapuerta M, Fernandez JR, Mora EF. Correlation for the estimation of the cetane number of biodiesel fuels and implications on the iodine number. Energy Policy 2009;37:4337–44.
[36] Supple B, Hildige H, Gomez EG, Leahy JJ. The effect of steam treating waste cooking oil on the yield of methyl ester. J Am Oil Chem Soc 2002;79: 175–8.
[37] Janaun J, Ellis N. Perspectives on biodiesel as a sustainable fuel. Renew Sustain Energy Rev 2010;14:1312–20.
[38] Sharma YC, Singh B, Upadhyay SN. Advancements in development and characterization of biodiesel: A review. Fuel 2008;87:2355–73.
[39] Demirbas A. Comparison of transesterification methods for production of biodiesel from vegetable oils and fat. Energy Conv Manage 2008;49:125–30.
[40] Toda M, Takagaki A, Okamura M, Kondo JN, Hayashi S, Domen K, et al. Biodiesel made with sugar catalyst. Nature 2005;438:178.
[41] Zhang S, Zu YG, Fu YJ, Luo M, Zhang DY, Efferth T. Rapid microwave-assisted transesterification of yellow horn oil to biodiesel using a heteropolyacid solid catalyst. Bioresour Technol 2010;101:931–6.
[42] Chen P, Min M, Chen Y, Wang L, Li Y, Chen Q, et al. Review of the biological and engineering aspects of algae to fuels approach. Int J Agric Biol Eng 2009;2:1–28.
[43] Guschina IA, Harwood JL. Lipids and lipid metabolism in eukaryotic alga. Prog Lipid Res 2006;45:160–86.
[44] Cardozo KH, Guaratini T, Barros MP, Falcao VR, Tonon AP, Lopes NP, et al. Metabolites from algae with economical impact. Comp Biochem Physiol C Toxicol Pharmacol 2007;146:60–78.
[45] Apt KE, Behrens PW. Commercial developments in microalgal biotechnology. J Phycol 1999;35:215–26.
[46] Tseng CK. Algal biotechnology industries and research activities in Chin. J Appl Phycol 2001;13:375–80.
[47] Cardozo KH, Guaratini T, Barros MP, Falcao VR, Tonon AP, Lopes NP, et al. Metabolites from algae with economical impac. Comp Biochem Physiol C Toxicol Pharmacol 2006.
[48] Richmond A. Handbook of microalgal culture. Biotechnol. Appl. Phycol. Blackwell Publishing; 2003.
[49] Barkley WCW, Lewin RA, Cheng L. Development of microalgal systems for the production of liquid fuels. In: Sadler T, editor. Villeneuve d'Ascq, France, Elsevier Appl. Sci.; 1987.
[50] Li Y, Horsman M, Wu N, Lan CQ, Dubois-Calero N. Biofuels from microalgae. Biotechnol Prog 2008;24:815–20.
[51] Richmond A. Microalgal biotechnology at the turn of the millennium: a personal view. J Appl Phycol 2000;12:441–51.
[52] Huntley ME, Redalje DG. CO_2 mitigation and renewable oil from photosynthetic microbes: a new appraisal. Mitigation Adapt Strat Global Change 2007;12:573–608.
[53] Chisti Y. Biodiesel from microalgae. Biotechnol Adv 2007;25:294–306.
[54] Lee YK. Microalgal mass culture systems and methods: their limitation and potential. J Appl Phycol 2001;13:307–15.
[55] Pulz O. Photobioreactors: production systems for phototrophic microorganisms. Appl Microbiol Biotechnol 2001;57:287–93.
[56] Carvalho AP, Meireles LA, Malcata FX. Microalgal reactors: a review of enclosed system designs and performances. Biotechnol Prog 2006;22:1490–506.
[57] Chaumont D. Biotechnology of algal biomass production: a review of systems for outdoor mass culture. J Appl Phycol 1993;5:593–604.
[58] Knuckey RM, Brown MR, Robert R, Frampton DMF. Production of microalgal concentrates by flocculation and their assessment as aquaculture feeds. Aquacult Eng 2006;35:300–13.
[59] Divakaran R, Pillai VNS. Flocculation of algae using chitosan. J Appl Phycol 2002;14:419–22.
[60] Molina Grima E, Belarbi EH, Acien Fernandez FG, Robles Medina A, Chisti Y. Recovery of microalgal biomass and metabolites: process options and economic. Biotechnol Adv 2003;20:491–515.
[61] Olaizola M. Commercial development of microalgal biotechnology: from the test tube to the marketplace. Biomol Eng 2003;20:459–66.
[62] Bosma R, Van Spronsen WA, Tramper J, Wijffels RH. Ultrasound, a new separation technique to harvest microalgae, J. Appl Phycol 2003;15:143–53.
[63] Millamena OM, Aujero EJ, Borlongan IG. Techniques on algae harvesting and preservation for use in culture and as larval food. Aquacult Eng 1990;9:295–304.
[64] Prakash J, Pushparaj B, Carlozzi P, Torzillo G, Montaini E, Materassi R. Microalgal biomass drying by a simple solar device. Int J Solar Energy 1997;18:303–11.
[65] Desmorieux H, Decaen N. Convective drying of Spirulina in thin layer. J Food Eng 2006;77:64–70.
[66] Leach G, Oliveira G, Morais R. Spray-drying of Dunaliella salina to produce a b-carotene rich powder. J Ind Microbiol Biotechnol 1998;20:82–5.
[67] Nindo CI, Tang J. Refractance window dehydration technology: a novel contact drying method. Drying Technol 2007;25:37–48.
[68] DunahayF T.G. Manipulation of microalgal lipid production using genetic engineering. Appl Biochem Biotechnol 1996;57–58:223–31.
[69] Pulz O, Gross W. Valuable products from biotechnology of microalgae. Appl Microbiol Biotechnol 2004;65:635–48.
[70] Lumbreras V, Stevens DR, Purton S. Efficient foreign gene expression in Chlamydomonas reinhardtii mediated by an endogenous intron. Plant J 1998;14, 441-147.
[71] Fuhrmann M. Expanding the molecular toolkit for *Chlamydomonas reinhardtii* – from history to new frontier. Protist 2002;153:357–64.
[72] Debuchy R, Purton S, Rochaix JD. The argininosuccinate lyase gene of *Chlamydomonasreinhardtii*: an important tool for nuclear transformation and for correlating the genetic and molecular maps of the ARG7 locus. EMBO J 1989;8:2803–9.
[73] Fernandez E, Schnell R, Ranum LP, Hussey SC, Silflow CD, Lefebvre PA. Isolation and characterization of the nitrate reductase structural gene of *Chlamydomonas reinhardtii*. Proc Natl Acad Sci USA 1989;86:6449–53.
[74] Boynton JE, et al. Chloroplast transformation in *Chlamydomonas* with high velocity microprojectiles. Science 1988;240:1534–8.
[75] Dunahay TG. Transformation of *Chlamydomonas reinhardtii* with silicon carbide whiskers. Biotechniques 1993;15:452–5.
[76] Randolph-Anderson BL, Boynton JE, Gillham NW, Harris EH, Johnson AM, Dorthu MP, et al. Further characterization of the respiratory deficient dum-1 mutation of *Chlamydomonas reinhardtii* and its use as a recipient for mitochondrial transformation. Mol Gen Genet 1993;236:235–44.
[77] Henry EC, Meints RH. Recombinant viruses as transformation vectors of marine macroalgae. J Appl Phycol 1994;6:247–53.
[78] Shimogawara K, Fujiwara S, Grossman A, Usuda H. High-efficiency transformation of *Chlamydomonas reinhardtii* by electroporation. Genetics 1998;148:1821–8.
[79] Kojima H, Kawata Y. A mini-transposon/transposase complex as a new tool for the genetic transformation of microalgae. Photosynthetic microorganisms in environmental biotechnology. In: (Kojima H, Lee YK, editors. Springer Verlag; 2001. p. 41–61.
[80] Kumar SV, Misquitta RW, Reddy VS, Rao BJ, Rajam MV. Genetic transformation of the green alga *Chlamydomonas reinhardtii* by *Agrobacterium tumefaciens*. Plant Sci 2004;166:731–8.
[81] Song D, Fu J, Shi D. Exploitation of oil-bearing microalgae for biodiesel. Chinese J Biotechnol 2008;24:341–50.
[82] Song DH, Liu J, Shi DJ. Expression of Humen Granulocyte Colony Stimulating Factor (G-CSF) in Synechococcus sp. PCC 7002. Dalian: The National Symposiums on Marine Biotechnology and Marine Drugs; 2006. p. 200–203.
[83] Kindle KL. High-frequency nuclear transformation of *Chlamydomonas reinhardtii*. Proc Natl Acad Sci USA 1990;87:1228–32.

[84] Benfey PN, Ren L, Chua NH. Tissue-specific expression from CaMV 35S enhancer subdomains in early stages of plant development. EMBO J 1990;9:1677–84.
[85] Ohresser M, René Matagne F, Loppes R. Expression of the arylsulphatase reporter gene under the control of the nit1 promoter in *Chlamydomonas reinhardtii*. Curr Genet 1997;31:264–71.
[86] Llamas A, Igeno MI, Galvan A, Fernandez E. Nitrate signalling on the nitrate reductase gene promoter depends directly on the activity of the nitrate transport systems in *Chlamydomonas*. Plant J 2002;30:261–71.
[87] Schroda M, Blöcker D, Beck CF. The HSP70 A promoter as a tool for the improved expression of transgenes in Chlamydomonas. Plant J 2000;21:121–31.
[88] Stevens DR, Purton S, Rochaix J-D. The bacterial phleomycin resistance gene ble as a dominant selectable marker in *Chlamydomonas*. Mol Gen Genet 1996;251:23–30.
[89] Cerutti H, Johnson AM, Gillham NW, Boynton JE. A eubacterial gene conferring spectinomycin resistance on *Chlamydomonas reinhardtii*: integration into the nuclear genome and gene expression. Genetics 1997;145:97–110.
[90] Sizova I, Fuhrmann M, Hegemann P. A Streptomyces rimosus aphVIII gene coding for a new type phosphotransferase provides stable antibiotic resistance to *Chlamydomonas reinhardtii*. Gene 2001;277:221–9.
[91] Stevens DR, Purton S. Genetic engineering of eukaryotic algae: progress and prospects. J Phycol 1997;33:713–22.
[92] Bateman JM, Purton S. Tools for chloroplast transformation in *Chlamydomonas*: expression vectors and a new dominant selectable marker. Mol Gen Genet 2000;263:404–10.
[93] Xiong L, Sayre RT. Engineering the chloroplast encoded proteins of *Chlamydomonas*. Photosynth Res 2004;80:411–9.
[94] Falciatore A, Bowler C. Revealing the molecular secrets of marine diatoms. Annu Rev Plant Biol 2002;53:109–30.
[95] Apt KE, Zaslavkaia L, Lippmeier JC, Lang M, Kilian O, Wetherbee R, et al. In vivo characterization of diatom multipartite plastid targeting signals. J Cell Sci 2002;115:4062–9.
[96] Lohuis MRT, Miller DJ. Genetic transformation of dinoflagellates (Amphidinium and Symbiodinium): expression of GUS in microalgae using heterologous promoter constructs. Plant J 1998;13:427–35.
[97] Chalfie M, Tu Y, Euskirchen G, Ward WW, Prasher DC. Green fluorescent protein as a marker for gene expression. Science 1994;263:802–5.
[98] Fuhrmann M, Hausherr A, Ferbitz L, Schodl T, Heitzer M, Hegemann P. Monitoring dynamic expression of nuclear genes in *Chlamydomonas reinhardtii* by using a synthetic luciferase reporter gene. Plant Mol Biol 2004;55:869–81.
[99] Mayfield SP, Franklin SE, Lerner RA. Expression and assembly of a fully active antibody in algae. Proc Natl Acad Sci USA 2003;100:438–42.
[100] Walker TL, Purdon S, Becker DK, Chris C. Microalgae as bioreactors. Plant Cell Rep 2005;24:629–41.
[101] Siripornadulsil S, Traina S, Verma DPS, Sayre RT. Molecular mechanisms of proline-mediated tolerance to toxic heavy metals in transgenic microalgae. Plant Cell 2002;14:2837–47.
[102] Zaslavskaia LA, Lippmeier JC, Shih C, Ehrhardt D, Grossman AR, Apt KE. Trophic conversion of an obligate photoautotrophic organism through metabolic engineering. Science 2001;292:2073–5.
[103] Sayre RT, Wagner RE, Siripornadulsil S, Farias C. Transgenic algae for delivering antigens to an animal, USA Patent Application. 2003/0211089.
[104] Melis A. Green alga hydrogen production: progress, challenges and prospects. Intl J Hydrogen Energ 2002;27:1217–28.
[105] Cai X-H, Brown C, Adhiya J, TrainaF S.J., Sayre RT. Growth and heavy metal binding properties of transgenic *Chlamydomonas* expressing a foreign metallothionein gene. Int J Phytoremediation 1999;1:53–65.
[106] Sun M, Qian K, Su N, Chang H, Lium J, Chen G. Foot-and-mouth disease virus VP1 protein fused with cholera toxin B subunit expressed in *Chlamydomonas reinhardtii* chloroplast. Biotechnol Lett 2003;25:1087–92.
[107] Takahama K, Matsuoka M, Nagahama K, Ogawa T. J Biosci Bioeng 2003;95:302.
[108] Schenk P, Thomas-Hall S, Stephens E, Marx U, Mussgnug J, Posten C, et al. Second generation biofuels: high-efficiency microalgae for biodiesel production. Bioenerg Res 2008;1:20–43.
[109] Margulies M, Egholm M, Altman WE, Attiya S, Bader JS, Bemben LA, et al. Genome sequencing in microfabricated high-density picolitre reactors. Nature 2005;437:376–80.
[110] Zhao T, Li GL, Mi SJ, Li S, Hannon GJ, Wang XJ, et al. A complex system of small RNAs in the unicellular green alga Chlamydomonas reinhardtii. Genes Dev 2007;21:1190–203.
[111] Riekhof WR, Sears BB, Benning C. Annotation of genes involved in glycerolipid biosynthesis in *Chlamydomonas reinhardtii*: discovery of the betaine lipid synthase BTA1(Cr). Eukaryot Cell 2005;4:242–52.
[112] Beer LL, Boyd ES, Peters JW, Posewitz MC. Engineering algae for biohydrogen and biofuel production. Curr Opin Biotechnol 2009;20:264–71.
[113] Davis MS, Solbiati J, Cronan JE. Overproduction of acetyl-CoA carboxylase activity increases the rate of fatty acid biosynthesis in *Escherichia coli*. J Biol Chem 2000;37:28593–8.
[114] Ohlrogge JB, Roesler KR, Shorrosh BS. Methods ofincreasing oil content of seeds. United States Patent. 5925805; 1999.
[115] Somers DA, Wyse DL, Gronwald JW. Transgenic plants expressing maize acetyl CoA carboxylase gene and method of altering oil content. United States Patent. 6222099; 2001.
[116] Roessler PG. Purification and characterization of acetyl-CoA carboxylase from the diatom *Cyclotella cryptica*. Plant Physiol 1990;92:73–8.
[117] Hu Q, Sommerfeld M, Jarvis E, Ghirardi M, Posewitz M, Seibert M, et al. Microalgal triacylglycerols as feedstocks for biofuel production: perspectives and advances. Plant J 2008;54:621–39.
[118] Moseley JL, Gonzalez-Ballester D, Pootakham W, Bailey S, Grossman: AR. Genetic interactions between regulators of *Chlamydomonas phosphorus* and sulfur deprivation responses. Genetics 2008;181:889–905.
[119] Sugimoto K, Midorikawa T, Tsuzuki M, Sato: N. Upregulation of PG synthesis on sulfur-starvation for PS I in Chlamydomonas. Biochem Biophys Res Commun 2008;369:660–5.
[120] Sheehan J, Dunahay T, Benemann J, Roessler PG. US Department of Energy's Office of Fuels Development, July 1998. A Look Back at the US Department of Energy's Aquatic Species Program – Biodiesel from Algae, Close Out Report TP-580-24190, Golden, CO: National Renew. Energ. Lab.; 1998.
[121] Shen YQ, Burger G. Plasticity of a key metabolic pathway in fungi, Funct. Integr. Genomics; 1998. p. 1–7.
[122] Verwoert II, Van Der Linden KH, Walsh MC, Nijkamp HJ, Stuitje AR. Modification of *Brassica napus* seed oil by expression of the *Escherichia coli* fabH gene, encoding 3-ketoacyl-acyl carrier protein synthase III. Plant Mol Biol Reptr 1995;27:875–86.
[123] Roesler K, Shintani D, Savage L, Boddupalli S, Ohlrogge J. Targeting of the Arabidopsis homomeric acetyl-coenzyme A carboxylase to plastids of rapeseeds. Plant Physiol 1997;113:75–81.
[124] Grotewold E. Transcription factors for predictive plant metabolic engineering: are we there yet? Curr Opin Biotechnol 2008;19:138–44.
[125] Reik A, Zhou Y, Collingwood TN, Warfe L, Bartsevich V, Kong Y, et al. Enhanced protein production by engineered zinc finger proteins. Biotechnol Bioeng 2007;97:1180–9.
[126] Michinaka Y, Shimauchi T, Aki T, Nakajima T, Kawamoto S, Shigeta S, et al. Extracellular secretion of free fatty acids by disruption of a fatty acyl-CoA synthetase gene in Saccharomyces cerevisiae. J Biosci Bioeng 2003;95: 435–40.
[127] Nojima Y, Kibayashi A, Matsuzaki H, Hatano T, Fukui S. Isolation and characterization of triacylglycerol-secreting mutant strain from yeast, Saccharomyces cerevisiae. J Gen Appl Microbiol 1999;45:1–6.
[128] Tietge UJF, Bakillah A, Maugeais C, Tsukamoto K, Hussain M, Rader DJ. Hepatic overexpression of microsomal triglyceride transferprotein (MTP) results in increased in vivo secretion of VLDL triglycerides and apolipoprotein B. J Lipid Res 1999;40:2134–9.
[129] Tietge UJF, Maugeais C, Cain W, Grass D, Glick JM, de Beer FC, et al. Overexpression of secretory phospholipase A2 causes rapid catabolism and altered tissue uptake of high density lipoprotein cholesteryl ester and apolipoprotein A-I. J Biol Chem 2000;275:10077–84.
[130] Janvilisri T, Venter H, Shahi S, Reuter G, Balakrishnan L, van Veen HW. Sterol transport by the human breast cancer resistance protein (ABCG2) expressed in Lactococcus lactis. J Biol Chem 2003;278:20645–51.
[131] Mentewab A, Stewart CN. Overexpression of an Arabidopsis thaliana ABC transporter confers kanamycin resistance to transgenic plants. Nat Biotechnol 2005;23:1177–80.
[132] Yu L, Gupta S, Xu F, Liverman ADB, Moschetta A, Mangelsdorf DJ, et al. Expression of ABCG5 and ABCG8 is required for regulation of biliary cholesterol secretion. J Biol Chem 2005;280:8742–7.
[133] Agriculture in Iran, From Wikipedia, the free encyclopaedia. Available from http://en.wikipedia.org/wiki/Agriculture_in_Iran [accessed 17.07.2010].
[134] Metting FB. Biodiversity and application of microalgae. J Ind Microbiol 1996;17:477–89.
[135] Hashemi Nejad M, Tabatabaei M, Mansourpanah Y, Khatami far M, Javani A. Upstream and downstream strategies to economize biodiesel production. Bioresour Technol 2011;102:461–8.
[136] Ghareyazie B, Alinia F, Menguito CA, Rubia LG, Palma JM, Liwanag EA, et al. Enhanced resistance to two stem borers in an aromatic rice containing a synthetic *cryIA*(*b*) gene. Mol Breeding 1997;3:401–14.
[137] Salehi Jozani GR, Komakhin RA, Piruzian ES. Comparative study of the expression of the native, modified, and hybrid *cry*3a genes of *Bacillus thuringiensis* in prokaryotic and eukaryotic cells. Russ J Genet 2005;41:116–21.
[138] Tohidfar M, Ghareyazie B, Mohammadi M. *Agrobacterium*-mediated transformation of cotton using a chitinase gene. Plant Cell Tissue Organ Cult 2005;83:83–96.
[139] Rajabi-Memari H, Jalali-Javaran M, Rasaee MJ, Rahbarizadeh F, Forouzandeh-Moghadam M, Esmaili A. Expression and characterization of a recombinant single-domain monoclonal antibody against MUC1 mucin in tbacco plants. Hybridoma 2006;25:209–15.
[140] Ismaili A, Jalali-Javaran M, Rasaee MJ, Rahbarizadeh F, Forouzandeh-Moghadam M, Memari HR. Production and characterization of anti-mucin (MUC1) single-domain antibody in tobacco (*Nicotiana tabacum* cultivar Xanthi). Biotechnol Appl Biochem 2007;47:11–9.
[141] Salehi Jouzani G, Irina V, Goldenkova E, Piruzian S. Expression of hybrid *cry*3aM-*lic*BM2 genes in transgenic potatoes (*Solanum tuberusom*). Plant Cell Tissue Organ Cult 2008;92:321–5.
[142] Tohidfar M, Salhi G. Genetic engineering of crop plants for enhanced resistance to insects and diseases in Iran. Transgenic Plant J 2008;2:151–6.
[143] Tohidfar M, Ghareyazie B, Mousavi M, Yazdani S. *Agrobacterium*-mediated transformation of cotton (*Gossypium hirsutum*) using a synthetic *cry1Ab* gene for enhanced resistance against *Heliothis armigera*. Iran J Biotechnol 2008;6:164–73.

[144] Zare N, Valizadeh M, Malbobi M, Tohidfar M. Regeneration of Iranian alfalfa by somatic embryo and shoot apex. Proceeding of the 2nd national congress of cellular and molecular biology, 29–30 January, Kermanshah, Iran; 2008. p. 335–7.
[145] Jafari M, Valizadeh M, Norouzi P, Malboobi MA, Ghareyazie B, Mohammadi SA, et al. Enhanced resistance to a lepidopteran pest in transgenic sugar beet plants expressing synthetic *cry1Ab* gene. Euphytica 2009;165:333–44.
[146] Bazargani MM, Tohidfar M, Ghareyazie B, Salehi Jouzani G, Sayed-Tabatabaei BE. Transformation of Iranian cotton varieties using shoot apex. Transgenic Plant Res 2010:107–12.
[147] Safarnejad MR, Fischer R, Commandeur U. Recombinant-antibody-mediated resistance against Tomato yellow leaf curl virus in *Nicotiana benthamiana*. Arch Virol 2009;154(3):457–67.
[148] Tohidfar M, Rasoly H, Hagnazary A, Ghareyazie B. Evaluation of stability of chitinase gene in Transgenic offspring of cotton (*Gossypium hirsutum*). Iran J Biotechnol 2009;7(1):45–50.
[149] Yazdanpanah F, Tohidfar M, Ashari M, Ghareyazi B, Mosavi M. Enhanced insect resistance to bollworm(*Helicoverpa armigera*) in cotton containing a synthetic *cry1Ab* gene. Ind J Biotechnol 2009;8:72–7.
[150] Zare N, Valizadeh M, Tohidfar M, Mohammadi SA, Malboobi MA, Habashi AA. Selection of regeneration genotypes from Iranian alfalfa cultivars. J Food Agric Environ 2009;7:567–72.
[151] Haghnazari A, Gavadi M, Tohidfar M. Zygosity identification in transgenic cotton (*Gossypium hirsutum*) by real time quantitative PCR. Euphytica; 2010, in press.
[152] Hadi MR, Shariati M, Afsharzadeh S. Microalgal biotechnology: carotenoid and glycerol production by the green algae *Dunaliella* isolated from the Gave-Khooni salt marsh. Iran Biotechnol Bioprocess Eng 2008;13(5): 540–4.